Utilization of Metagenomics for Evaluation of Three Enrichment Procedures for Detection and Isolation of *E. coli* O157:H7 in Mung Bean Sprout Irrigation Water

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SUMMARY

Introduction

Spent sprout irrigation water (SSIW) has been tested in compliance programs for detection of *E. coli* O157:H7 in sprouts. However, detection and isolation by standard cultural methods can be difficult due to the high background microflora associated with this matrix.

Purpose

This study used enriched SSIW microbiomes to evaluate three procedures for rapid detection and isolation of *E. coli* O157:H7 from artificially contaminated mung bean SSIW samples.

Methods

Mung bean SSIW inoculated with *E. coli* O157:H7 at low (0.075 cfu/mL) and high (0.75 cfu/mL) levels, was stored at 4°C for 72 h. Three enrichment procedures were used: (1) FDA BAM procedure using static incubation in mBPWp media for 5 h at 37ºC, followed by addition of acriflavine (A), cefsulodin (C), and vancomycin (V) and then further incubation with no shaking at 42ºC to enhance selectivity; (2) mBPWp with CV at 42°C with shaking, and (3) mBPWp with CV at 42°C without shaking. SSIW prior to enrichment along with three biological replicate samples at each inoculum level for the three methods were collected for shotgun metagenomic analysis after enrichment. Sequencing was accomplished using Illumina technology and a k-mer based method was used for bacterial taxa identification.

Results

The relative abundance of *E. coli* O157:H7 at the 24 h enrichment time point for both the low and high inoculum was found to be between 15 and 35 % for test conditions (2) and (3) but less than 3 % for test condition (1). Both modifications to the BAM procedure resulted in lower levels of *Enterobacter* and *Pseudomonas* in the SSIW microbiomes, along with lesser relative abundance differences in other taxa among the enrichment procedures.

Significance

This study demonstrates how metagenomics provides an additional tool for enrichment method development studies that can be used to improve current cultural methods used for pathogen detection in difficult matrices.

INTRODUCTION

Detection of *E. coli* O157:H7 in alfalfa sprouts by standard cultural methods is time consuming and can be difficult due to the high background flora (Weagant et al. 1995). A procedure for the enrichment of Shiga toxin producing *E. coli* in foods using selective enrichment of samples in modified Buffered Peptone Water (mBPWp) (Weagant & Bound, 2001) was combined with detection by the FDA BAM real-time PCR procedure, specific for *stx*1, *stx*2, and the O157wzy using the ABI7500 (Feng et al, 2017). for detection of EHEC in sprout water samples.

In a previous study, we found that addition of antibiotics (CV) and elevated incubation temperature (42°C) with shaking (140 rpm) led to the best recovery of low levels of target *E. coli* O157:H7 from inoculated alfalfa sprouts over 5 strains tested. These results show that the enrichment procedure is reliable, and can provide rapid results when combined with immunomagnetic separation (IMS) and real-time PCR. (Weagant et al., 2011). We refer to this method as Shaking mBPWp + CV (S+CV). We also examined the use of the mBPWp+CV broth enrichment at 42°C without shaking (NS+CV). Previous work has also shown that the use of immunomagnetic separation and acid treatment of sprout enrichment cultures and enrichments from other produce with a high background flora improved isolation of *E. coli* O157:H7 (Fedio et al. 2012; Yoshitomi et al., 2012).

The current procedures for evaluation of qualitative detection methods for pathogens in foods used by the FDA require that samples are inoculated with the target organism and results that show fractional recovery of the organism are used for statistical analysis and determination of method efficacy (FDA, 2019).

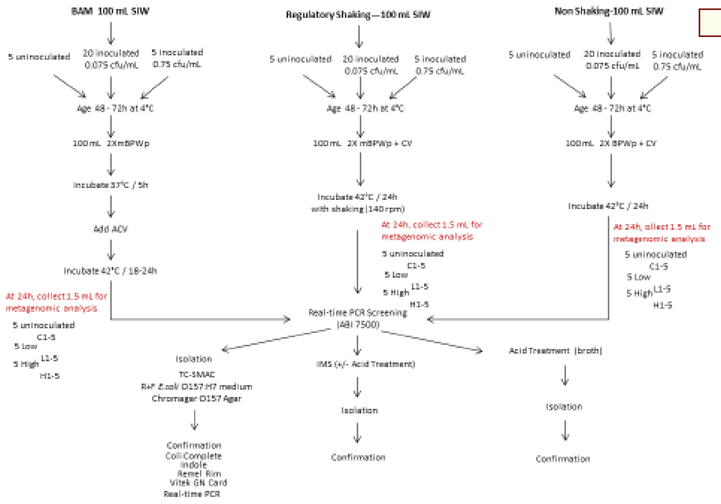

Figure 1: Procedures for enriching sprout irrigation water for *E. coli* O157:H7: BAM, Shaking (S+CV), and Non-Shaking (NS+CV).

BAM	PCR	Plating	Plating + Acid	IMS	IMS + Acid
C1	-	-	-	-	-
C2	-	-	-	-	-
C3	-	-	-	-	-
C4	-	-	-	-	-
C5	-	-	-	-	-
	0/5	0/5	0/5	0/5	0/5
L1	+	+	+	+	+
L2	+	+	-	+	+
L3	+	+	+	+	+
L4	+	+	+	+	+
L5	+	+	+	+	+
L6	-	-	-	-	-
L7	+	+	+	+	+
L8	+	+	+	+	+
L9	+	-	+	+	+
L10	+	+	+	+	+
L11	+	-	+	+	+
L12	+	+	+	+	+
L13	+	+	+	+	+
L14	+	-	+	+	+
L15	+	+	+	+	+
L16	+	+	+	+	+
L17	-	-	-	-	-
L18	+	+	+	+	+
L19	+	+	+	+	+
L20	+	+	+	+	+
	18/20	15/20	17/20	18/20	18/20
H1	+	-	+	-	+
H2	+	+	+	+	+
H3	+	+	+	-	+
H4	+	-	+	+	+
H5	+	+	+	+	+

RS	PCR	Plating	Plating + Acid	IMS	IMS + Acid
C1	-	-	-	-	-
C2	-	-	-	-	-
C3	-	-	-	-	-
C4	-	-	-	-	-
C5	-	-	-	-	-
	0/5	0/5	0/5	0/5	0/5
L1	+	+	+	+	+
L2	+	+	+	+	+
L3	+	+	+	+	+
L4	+	+	+	+	+
L5	+	+	+	+	+
L6	+	+	+	+	+
L7	+	+	+	+	+
L8	+	+	+	+	+
L9	+	+	+	+	+
L10	+	+	+	+	+
L11	+	+	+	+	+
L12	+	+	+	+	+
L13	+	+	+	+	+
L14	+	+	+	+	+
L15	+	+	+	+	+
L16	+	+	+	+	+
L17	+	+	+	+	+
L18	+	+	+	+	+
L19	+	+	+	+	+
L20	+	+	+	+	+
	19/20	19/20	20/20	19/20	20/20
H1	+	+	+	+	+
H2	+	+	+	+	+
H3	+	+	+	+	+
H4	+	+	+	+	+
H5	+	+	+	+	+

NS	PCR	Plating	Plating + Acid	IMS	IMS + Acid
C1	-	-	-	-	-
C2	-	-	-	-	-
C3	-	-	-	-	-
C4	-	-	-	-	-
C5	-	-	-	-	-
	0/5	0/5	0/5	0/5	0/5
L1	+	+	+	+	+
L2	+	+	+	+	+
L3	-	-	-	-	-
L4	+	+	+	+	+
L5	+	+	+	+	+
L6	+	+	+	+	+
L7	-	-	-	-	-
L8	+	+	+	+	+
L9	+	+	+	+	+
L10	+	+	+	+	+
L11	+	+	+	+	+
L12	+	+	+	+	+
L13	-	-	-	-	-
L14	+	+	+	+	+
L15	+	+	+	+	+
L16	+	+	+	+	+
L17	+	+	+	+	+
L18	+	+	+	+	+
L19	+	+	+	+	+
L20	+	+	+	+	+
	15/20	15/20	15/20	15/20	15/20
H1	+	+	+	+	+
H2	+	+	+	+	+
H3	+	+	+	+	+
H4	+	+	+	+	+
H5	+	+	+	+	+

Table 2: Isolation of E. coli O157:H7 from artificially contaminated sprouts by BAM, S+CV, and NS+CV procedures. No significant difference (p=0.05) was observed between the procedures X^2<3.84.

MATERIALS AND METHODS

Inoculation Procedure:

Both the inoculated mung bean SIW and uninoculated mung bean SIW were shipped to the NMSU FSL from the FDA (Moffet Center, Bedford Park, IL). Samples were aged before enrichment during transport (at < 4°C for < 72h). SIW samples were weighed (100 g portions) into sterile polythene filter bags. Uninoculated SIW samples were used as controls. For inoculated SIW, samples were inoculated with an appropriate volume of a 1/10 dilution of a Butterfields Phosphate Buffer culture of *E. coli* O157:H7 strain H3482 in order to achieve the low (0.017 cfu/mL) and high (0.17 cfu/mL) inoculation levels. Inoculum was calculated by plate counts on the culture used for inoculating SIW at the Moffet Center and confirmed by MPN at the NMSU FSL.

Enrichment Procedures:

Three enrichment procedures were examined:

1. **BAM procedure**: For each 100 g sample, 100 mL of pre-warmed 2 X mBPWp was added. Samples were stomached for 2 min and incubated for 5 h at 37°C. Acriflavin (10 mg/L), cefsulodin (10 mg/L), and vancomycin (8 mg/L), were then added and the samples were incubated at 42°C for a total of 20- 24 h .
2. **S+CV Method**: For each 100 g sample, 100 mL of pre-warmed 2 X mBPWp containing cefsulodin (10 mg/L) and vancomycin (8 mg/L) were added and the samples were incubated at 42°C with shaking at 140 rpm for 24 h.
3. **NS+CV Method**: For each 100 g sample, 225 mL of pre-warmed mBPWp containing cefsulodin (10 mg/L) and vancomycin (8 mg/L) were added and the samples were incubated at 42°C (static) for 24 h.

***E. coli* O157:H7 detection, isolation and confirmation:**

As shown in Figure 1, E. *coli* O157:H7 was detected by PCR analysis on the ABI 7500 FAST for *stx*1, *stx*2, O157wzy markers, plating on selective media (CHROMagar O157, R&F *E. coli* O157 Medium, and TC-SMAC),, by immunomagnetic separation with Dynabeads O157 with streaking on selective media and following acid treatment of broth or IMS beads as described by Fedio et al., 2012. Presumptive positive isolates were confirmed as described in the FDA BAM (Feng et al., 2017.)

Metagenomic Sequencing and Analysis:

Genomic DNA was extracted from pellets of the 24 h enrichments and used to prepare shotgun metagenomic sequencing libraries using the Illumina Nextera DNA Flex Library Prep Kit. Paired-end 2 x 250 bp sequencing was performed using an Illumina MiSeq. The bacterial community was determined using a custom Python program that utilizes a k-mer method for taxonomic identification. Sequence reads were trimmed using CLC Genomics Workbench and then queried for *E. coli* serotyping and virulence genes using BLAST.

Reference to any commercial materials, equipment, or process does not in any way constitute approval, endorsement, or recommendation by the U.S. Food and Drug Administration

RESULTS

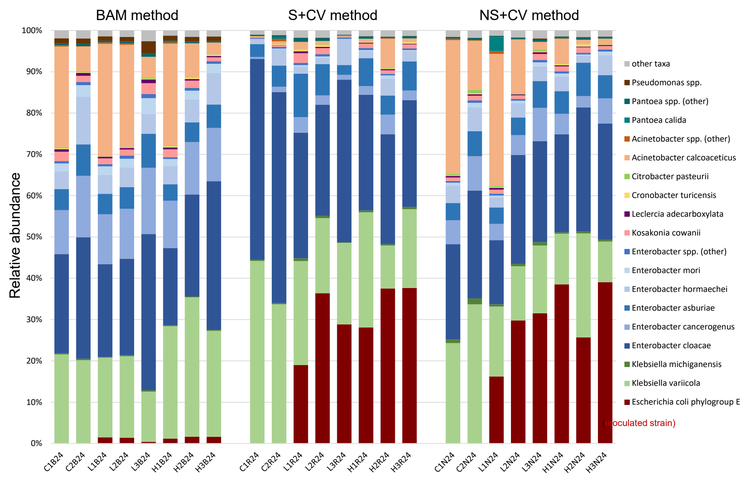

Figure 2: Microbiomes of enriched mung bean sprout irrigation water. Control samples (C) were uninoculated, Low inoculum samples (L) were inoculated with 0.075cfu/mL of *E.coli* O157:H7 and High inoculum samples ((H) with 0.75 cfu /mL of *E. coli* O157:H7

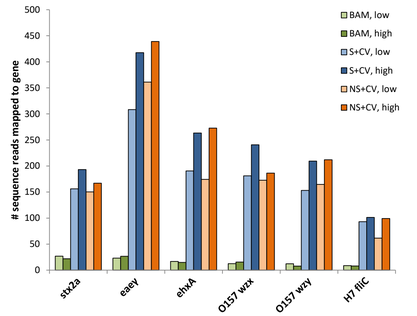

Figure 3: Detection of serotyping and virulence genes in the metagenomic datasets. The number of sequence reads matching the respective genes were normalized to a total of 2.5 million reads per sample and averaged for each enrichment procedure and inoculum level.

DISCUSSION

- When sprout water was inoculated with 0.075 cfu *E. coli* 0157:H7/mL, none of the test conditions were shown to differ significantly for detection/isolation of the pathogen.
- It is important to mention that the BAM enriched samples had considerably fewer "typical" isolates on the selective agars and often required restreaking of the sample on selective media to obtain isolated colonies.
- The background microflora of the SIW was determined on plate count agar to be 1 X 10^6 cfu/mL
- Analysis of the microbiome of enriched mung bean SIW samples revealed that the relative abundance of *E. coli* O157:H7 at the 24 h enrichment time point for both the low and high inoculum was found to be between 15 and 35 % for S + CV and NS + CV samples but less than 3 % for samples enriched by the BAM procedure (Figure 2).
- The S + SV and NS + CV methods enhanced the ability of the inoculated *E. coli* O157:H7 to compete with *Enterobacter* and *Pseudomonas* for growth compared to the BAM method.
- Consistent with the bacterial community analysis, detection of genes in the metagenomic datasets that are used to determine STEC pathogenicity demonstrated that the S + CV and NS + CV methods provide comparable and superior detection sensitivity over the BAM method (Figure 3).
- Additional studies were conducted to obtain fractional recovery of the inoculated organisms in the sprout water and it was found that inoculation at 0.017 cfu/mL gave results that were suitable for statistical analysis (Fedio et al., 2020).

CONCLUSIONS

- Overall, the S+CV and NS+CV procedures performed better than the BAM method for detection of *E. coli* O157:H7 in artificially contaminated mung bean sprout irrigation water.
- Metagenomics provides an additional tool for enrichment method development studies that can be used to improve current cultural methods used for pathogen detection in difficult matrices.

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